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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/566, 33/573 — A1

(11) International Publication Number: WO 96/38728

(43) International Publication Date: 5 December 1996 (05.12.96)

(21) International Application Number: PCT/EP96/02348

(22) International Filing Date: 1 June 1996 (01.06.96)

(30) Priority Data:
MI95A001155 2 June 1995 (02.06.95) IT

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD FOR MEASURING THE A2a RECEPTOR BINDING ACTIVITY OF COMPOUNDS OF PHARMACOLOGICAL INTEREST BY THE USE OF THE TRITIATED LIGAND (^{3}H)-SCH 58261

(57) Abstract

The invention relates to a method for evaluating the adenosine A2a receptor binding affinity of compounds of pharmacological interest. Moreover, the invention relates to reagents and a kit particularly suitable for the above mentioned purpose.

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A METHOD FOR MEASURING THE A2a RECEPTOR BINDING ACTIVITY OF COMPOUNDS OF PHARMACOLOGICAL INTEREST BY THE USE OF THE TRITIATED LIGAND (^{3}H)-SCH 58261

PURPOSE THE INVENTION

Adenosine modulates a wide range of physiological functions by interaction with different receptor subtypes named A1, A2a, A2b and A3 [Pharmacol. Rev., 46, 143, (1994)]. While the availability of A1 receptor ligands led to rapid progress in the characterization of this receptor subtype, the pharmacology of A2a adenosine receptors was hampered by the lack of selective ligands [Med. Res. Rev., 12, 423, (1992)]. In the past, using different strategies to block the interaction with A1 receptors [Naunyn-Schmiedeberg's Arch. Pharmacol., 325, 218, (1984); Mol. Pharmacol., 29, 331, (1986], the non selective agonist radioligand (^{3}H)-5'-N ethylcarboxamidoadenosine [(^{3}H)-NECA] has been used successfully to label the A2a adenosine receptor in rat striatal membranes [Mol. Pharmacol., 29, 331, (1986)]. However, [(^{3}H)-NECA] has been also reported to interact with non receptors binding proteins in both cerebral and peripheral tissues [Annu. Rev. Pharmacol. Toxicol., 27, 315, (1987)]. More recently, the compound 2-[p-(2-carboxyethyl)-phenethyl-amino]-5'-N-ethylcarboxyadenosine (CGS 21680), a NECA derivative with high affinity (Ki=14 nM) and selectivity (A1 vs A2a ratio of about 180-fold) for A2a adenosine receptors, has become the radioligand of choice to investigate this receptor subtype [J. Pharmacol. Exp. Ther., 251, 888, (1989)].

The development of A2a antagonist radioligands has been hampered by the lack of selective compounds. Although compound (8-[4-[[[[2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (XAC) is a moderately A1 selective antagonist, it was used as labeled compound to characterize the A2a adenosine receptor in human platelet membranes [FEBS Lett., 199, 269, (1986)]. However, the specific binding of (^{3}H)XAC to platelet membranes was only 40% of the total binding. Like NECA, PD 115119, a sulphonamide congener of 1,3-diethyl-8-phenylxanthine, is equiactive at A1 and A2a receptors. In the presence of 20 nM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), (^{3}H)-PD 115119 interacted specifically with A2a striatal receptors, but its radiostability was found to be poor [Naunyn-Schmiedeberg's Arch. Pharmacol., 335, 64, (1987)]. Recently, the A2a selective antagonist (^{3}H)-(E,18%-Z,82%)-8-(3,4-dimethoxystyryl)-1,3-dipropylxanthine [(^{3}H)-KF 17837S] has been indicated to interact directly with the A2a adenosine receptor in rat striatal tissue, showing a specific binding of 60-70% [Mol. Pharmacol., 46, 817, (1995)]. However, although KF 17837S was described to be a potent A2a antagonist (Ki=7.8 nM) and selective (A1/A2a=49) in the original work [J. Med. Chem., 36, 3731, (1993)], substantial differences in A2a affinity (Ki values ranging from 30 to 60 nM) and selectivity (A1/A2a=19) have been reported [J. Med. Chem., 36, 1333, (1993); Br. J. Pharmacol. 112, 659, (1994)]. Recently, the compound 5-amino-7-(2-phenetyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine was described

as the first potent (Ki=2,3 nM) and selective (A1/A2=53) non-xanthine A2a antagonist [Bioorg. Med. Chem. Lett., 4, 2539, (1994)]. The labeled form of the compound appears ideally suited for the characterization of the A2a adenosine receptor and for the identification of new compounds interacting with this receptor subtype.

SUMMARY OF THE INVENTION

One aspect of the present invention is the preparation of the labeled compound 5-amino-7-[2-(2',4',5' ^{3}H)phenetyl]-2-(2-furyl)-pyrazolo-[4,3-e]--1,2,4-triazolo[1,5-c]pyrimidine (hereinafter referred to as (^{3}H)-Compound).

The preferred label of the invention has the purpose of facilitating the measurement of the relative binding affinity values, preferably by introducing tritium (^{3}H) atoms, and more preferably, located on the phenethyl group at the positions 2', 4' and 5'.

The second aspect of the invention is a method for determining the adenosine A2a receptor binding affinity of a test compound; said method consisting in:

(a) preparing purified mammalian brain tissue containing A2a receptors;

(b) adding (^{3}H)-Compound to said mammalian brain tissue;

(e) adding a test compound to said mammalian brain tissue; and

(d) measuring the amount of radioligand complexed with said A2a receptors.

A test compound may be synthesized and/or purified from natural source such as animal or plant tissue.

The third aspect of the invention is a kit for determining the A2a binding activity of a test compound; said kit comprising:

(a) a sample of purified mammalian brain tissue containing A2a receptors; and

(b) a sufficient amount of labeled compound to determine the level of A2a receptor affinity of said test compound.

DETAILED DESCRIPTION OF THE INVENTION

Because of the potential utility of having an A2a antagonist radioligand, the (^{3}H)-Compound was synthesized. The studies described here were performed in order to characterize binding properties of (^{3}H)-Compound to A2a receptors of rat striatum.

Synthesis of ^{3}H-Compound

(^{3}H)-Compound was obtained by reduction with tritium gas in the presence of 10% Pd/C (Dupont-New England Nuclear, Boston, MA, USA) from the precursor 5-amino-7-[2-(2',4',5'-tribromo)-phenylethyl]-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine. The final product was purified by HPLC to give the title (^{3}H)-Compound with radiochemical purity of 99% and specific activity of 68.6 Ci/mmol.

Tissue Preparation

Male Sprague-Dawley rats (Charles-River, Calco, Italy) weighing 250-300 g were sacrificed by decapitation and striatum was dissected on ice. The tissue was homogenised in a Polytron PTA 10 Probe (setting 5, 20 sec) in 25 volumes (v/v) of 50 mM Tris-HCl buffer, pH 7,4, centrifuged at 48,000 x g for 10 min at 4°C and resuspended in Tris-HCl containing 2

units/ml of adenosine deaminase. After 30 min of incubation at 37°C, the membranes were centrifuged and pellet was stored at -70°C.

Binding Assay

Saturation binding experiments were carried out in polypropylene test tubes containing an aliquot of striatal membranes (100 ug of protein /assay) in incubation buffer (50 mM Tris-HCl, pH 7.4) and 11 different concentrations of (^{3}H)-Compound (0.0625-64 nM), in a final volume of 0.5 ml Non specific binding was defined in the presence of NECA 50 uM. All assays were performed at 25°C for 30 min, the separation of the free radioligand from the one bound to the receptor was carried out by fast filtration through Whatman GF/B filters using the Brandel cell harvester (Gaithersburg, MD, USA). Filters were washed twice with ice cold buffer (5 ml) and placed in vials containing 5 ml of scintillation liquid (Ready Safe, Beckman Instruments, Fullerton, CA, USA). Radioactivity was measured using a LS-6000 Beckman liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA) with an efficiency of 50 to 60%. Protein concentration was determined by the method of Lowry [J. Biol. Chem., 193, 265, (1951)] using bovine serum albumin as standard.

In the competition studies, different concentrations of several adenosine receptor agonists and antagonists were included in the incubation buffer containing 0.2 nM (^{3}H)-Compound.

Binding parameters were estimated by using the computerized program LIGAND [Anal. Biochem., 107, 220, (1980)].

Results

After incubation at 25 °C and pH 7.4, 0.2 nM (^{3}H)-Compound bound to rat striatal membranes with a specific binding of 92%, which increased linearly with respect to protein concentration over a range of 50-300 ug of protein/assay. The presence of 10 mM $MgCl_2$ or 100 uM guanosine triphosphate (GTP) in the assay mixture did not modified significantly the percentage of specific binding.

The reaction kinetic showed that (^{3}H)-Compound binding reached equilibrium after approximately 5 min. and was stable for at least 4 hr. (^{3}H)-Compound binding was rapidly reversed by the addition of NECA 50 uM. Association and dissociation rate constants were the following: K_{obs}=0.85/min and K_{-1}=0.62/min from a $T_{1/2}$=1.12 min. A dissociation constant (Kd) value of 0.54 nM was calculated from these experiments.

Saturation experiments showed that (^{3}H)-Compound bound to a single class of receptors in rat striatal membranes, with Kd and apparent number of receptors (Bmax) values of 0.70 nM and 971 fmol/mg of protein, respectively.

In the competition experiments, several adenosine receptor agonists inhibited the (^{3}H)-Compound binding to rat striatal membranes with the following order of potency: NECA ≥ CGS 21680 > 2-phenylaminoadenosine (CV 1808) > R-N^6-2-phenylisopropyladenosine (R-PIA) > cyclohexyladenosine (CHA) > S-N^6-2-phenylisopropyl-adenosine (S-PIA). The non selective agonist NECA proved to be the most potent compound with nanomolar affinity (Ki=61 nM). Moreover, the A1 selective agonist

R-PIA was found about 8-fold more potent than its stereoisomer S-PIA, thus showing the stereoselectiviq of (^{3}H)-Compound binding. The ability of several xanthine and non-xanthine adenosine receptor antagonists in competing (^{3}H)-Compound in the binding to A2a striatal receptors was also examined. Their order of potency was: CGS 15943 > Compound > XAC = KF 17837 > DPCPX. OGS 15943 was the most potent compound in inhibiting (^{3}H)-Compound binding with a Ki value of 0.38 nM.

(^{3}H)-Compound interacted with adenosine agonists and antagonists with an order of potency similar to that observed using (^{3}H)-CGS 21680 as the radioligand, and it showed a selective interaction with the A2a receptor [J. Pharmacol. Exp. Ther., 251, 888, (1989)].

Conclusion

In conclusion, (^{3}H)-Compound, labeling directly the adenosine A2a striatal receptor, proves to be an excellent means for studying this adenosine A2a receptor subtype in mammalian brain. Clear advantages over other A2a antagonist radioligand proposed for this purpose are the high receptor affinity and the low non specific binding. A kit containing the necessary components to perform the assays as described above, as well as a method to utilize such kit, can be considered essential for evaluating the interaction of a test compound with A2a adenosine receptors in mammalian brain tissues. Moreover, the (^{3}H)-Compound has the characteristics to become a useful tool for the investigation of A2a receptors distributed in peripheral tissues, such as vascular preparations,

platelets and neutrophils, in which their presence has been clearly demonstrated [TiPS, 14, 360, (1993)].

CLAIMS

1. A method for determining the adenosine A2a receptor binding affinity of compounds of pharmacological interest, said method consisting in:

(a) preparing purified mammalian brain tissue containing A2a receptors;

(b) adding the labeled form of the compound 5-amino-7-(2-phenetyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine to said mammalian brain tissue;

(e) adding a test compound to said mammalian brain tissue; and

(d) measuring the amount of radioligand complexed with said A2a receptors.

2. The method according to claim , wherein the label consists in an enriched level of radioactive atoms.

3. The method according to claim 2, wherein the label consists in an enriched level of tritium atoms.

4. The method according to claim 1, wherein said mammalian brain tissue is rat brain tissue.

5. A kit for determining the A2a binding affinity of a test compound, said kit comprising:

(a) a sample of purified mammalian brain tissue containing A2a receptors; and

(b) a sufficient amount of labeled form of the compound 5-amino-7-(2-phenetyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine to determine the A2a receptor affinity of said test compound.

6. A kit according to claim 5 containing 5-amino-7-(2-phenetyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine.

INTERNATIONAL SEARCH REPORT

International Application No
PC EP 96/02348

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/566 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, vol. 4, no. 21, 1 January 1994, LONDON UK, pages 2539-2544, XP000603773 P.G. BARALDI ET AL.: "Synthesis of new pyrazolo(4,3-e)1,2,4-triazolo(1,5-c) pyrimidine and 1,2,3-triazolo(4,5-c)pyrimidine displaying potent and selective activity as A2a adenosine receptor antagonists." see table 1 --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	RESEARCH COMMUNICATIONS IN MOLECULAR PATHOLOGY AND PHARMACOLOGY, vol. 87, no. 1, 1 January 1995, WSETBURY NY USA, pages 87-88, XP000603784 A. NEGRETTI ET AL.: "In vitro pharmacological profile of the new non-xanthine A2a adenosine antagonist 8FB-PTP" see the whole document ---	1-6
T	BRITISH JOURNAL OF PHARMACOLOGY, vol. 117, no. 7, 1 April 1996, LONDON UK, pages 1381-1386, XP000603818 C. ZOCCHI ET AL.: "Binding of the radioligand (3H)-SCH 58261, a new non-xanthine A2a adenosine receptor antagonist, to rat striatal membranes" see the whole document -----	1-6

1